Supplementary Methods

NHE3 Activity Determined by Two-Photon Microscopy:

Na^+/H^+ Exchange Activity assays in intact mouse jejunum was determined as rates of Na^+- dependent alkalinization using a two-photon microscope (MRC-1024MP, Bio-Rad, Hercules, CA) powered by a wide band, infrared (780 nm) combined photo-diode pump laser and mode-locked titanium-sapphire laser (Tsunami Ti:Sa laser, Spectra-Physics, Mountain View, CA) with a 60×/1.00 water immersion objective (Nikon), using the intracellular pH indicator, SNARF®-4F(5-(and-6)-carboxylic acid acetoxymethyl ester) (Invitrogen S-23920, Carlsbad, CA), modified from our previously report [21]. SNARF- 4F exhibits a significant pH-dependent emission shift which allows pHi to be determined from the ratio of the fluorescence intensities from two emission wavelengths, 580 nm and 640 nm at one excitation (780 nm, two photon excitation).

After 24 hours fasting (no food but ad libitum access to water), mice were sacrificed by an overdose of isoflurane. The abdomens were immediately opened by midline incision and proximal jejunums (upper 50% of small intestine starting 1 cm distal to ligament of Treitz) were excised and placed immediately in cold "Na buffer" (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM MgSO4, 1 mM $NaH2PO_4$, 25 mM mannose, and 20 mM HEPES, pH 7.4) and opened along the mesenteric border. Tissue strips were fixed to a glass coverslip with Krazy Glue (Elmer's Products Inc., Columbus, OH) with the mucosal surface facing up. All preparations were performed on ice. Full thickness jejunum was loaded with 20 μM SNARF-4F in Na^+ buffer at 37°C for 35 min with 95% O2, 5% CO2 gassing. Then the tissue was mounted into a perfusion chamber (RC-21BDW, Warner Instrument) with temperature controlled at 25°C. After sequential acidification with NH_4Cl prepulse (60 mM NH_4Cl in 78 mM Na^+ solution) for 20 min and Na^+-free buffer (TMA, 138 mM trimethylamine hydrochloride instead of Na^+ in Na buffer) for 20 min, mouse jejunum was perfused with Na solution containing either 25 mM D-mannose (Sigma M2069, St. Louis, MO) or 25 mM alpha-methyl-D-glucose (α-MD-G) (Sigma M9370, St. Louis, MO) for 12 min. Following this perfusion, the K^+ /nigericin (10 uM) method was used to calibrate the pH_i for each tissue. K^+ clamp buffer contained 20 mM HEPES, 20 mM MES, 75 mM KCl, 35 mM K gluconate, 14 mM Na gluconate, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 2 mM TMA-Cl, adjusted to pH 6.2, 6.8, 7.4. 1 mM probenecid (Sigma P8761, St. Louis, MO) was in all solutions to prevent SNARF-4F leakage. 50 μM HOE694 (Sonafi-Aventis, Bridgewater, NJ) was added in both NH_4Cl and TMA solutions to inhibit NHE1 and NHE2.

We studied epithelial cells 20~30 μm from the villus tip to avoid dying cells. Three images were collected at the end of the perfusion with initial Na, NH_4 and TMA solutions, and starting 1 min after switching the medium from TMA to Na with mannose or glucose, images were continuously recorded every 20 seconds for 5 min and then one image was taken every minute during the following 7 min. The images were stored and analyzed off line using MetaMorph 5.0 software (Roper Industries, Marlow, UK). Twenty areas of interest were randomly chosen in each case, and the same areas of interest were studied at the different time points from one mouse. The ratio of average fluorescence intensity in jejunum was converted to intracellular pH using a three point internal calibration. The initial slope of alkalization (ΔpH/min) (the maximum slope based on three continuous

time points over the first ~ 2 min was defined as the initial rate) was calculated and is referred to as the initial NHE3 activity.

Apical Cell surface Biiotinylation of NHE3 in Caco-2/bbe Cells:

NHS-SS-biotin was used to determine the amount of BB NHE3. Polarized Caco-2/bbe-SGLT1 cells seeded on 0.4 μm polycarbonate membrane Transwell semi-permeable supports were infected with adenoviral HA-NHE3 12 days post confluence. 48 hours later, the cells were serum starved for 4 h and then acidified by NH_4Cl prepulse/mannose (1 hour), The cells were then incubated with isotonic Na^+ solution containing 25 mM mannose or α-MD-G for 5 min. Cells were rinsed three times with ice-cold phosphate-buffered saline (150 mM NaCl and 20 mM Na_2HPO_4, pH 7.4) and twice in borate buffer (154 mM NaCl, 1.0 mM boric acid, 7.2 mM KCl, and 1.8 mM $CaCl_2$, pH 8.0). For surface labeling of NHE3, cells were incubated twice with 1.0 mg/ml NHS-SS-biotin (biotinylation solution; Pierce Chemical, Rockford, IL) for 40 min (on ice). Quenching buffers (20 mM Tris and 120 mM NaCl, pH 7.4) then were used to scavenge the unreacted biotin. Cells were washed three times with ice-cold PBS and solubilized with 0.7 ml N^+ buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na_3EDTA, 3 mM EGTA, and 1% Triton X-100). Of the 0.7 ml of cell lysate, 0.6 ml was incubated with Streptavidin-agarose beads (Pierce Chemical, Rockford, IL) to isolate the biotinylated proteins via 3 hour precipitation. The supernatant was retained as the intracellular fraction. The Streptavidin-agarose beads were washed five times in N^+ buffer (containing 0.1% Triton X-100) to remove nonspecifically bound proteins, and then 70 ul of loading butter (5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 1% 2-mercaptoethanol), was used to elute biotinylated proteins from beads. Two dilutions (20 ul and 40 ul) of total cell lysate, surface and intracellular proteins for each group were loaded, size-fractionated by SDS-PAGE (10% gels), and then electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk, the blots were probed with a monoclonal anti-HA antibody (Covance 16B12, Princeton, NJ), with normalization to actin using monoclonal anti-β-actin antibody (Sigma A2228) . Anti-GAPDH (U.S. Biological, Swampscott, MA) was used as a loading control.

NHERF1/2 shRNA Knock Down and Adenoviral HA-NHE3 Infection in Caco2-bbe Cells:

Lentiviral based shRNA was used to knock down NHERF1 and NHERF2 in Caco-2/bbe-SGLT1 cells, creating stable cell lines expressing the shRNAs over at least 10 passages. (Sarker et al, manuscript in preparation). 3 gene sequences for shRNA clones specific for NHERF1 and NHERF2 in the lentivirus plasmid vector pLKO.1-puromycin were obtained through the Johns Hopkins HiT center from Open Biosystems (Huntsville, AL). The negative control was a lenti shRNA construct specific to GFP which is not expressed in mammalian cells endogenously. These constructs were used to generate lentiviral transduction particles as described [13]. Puromycin selection (10 μg/ml) allowed generation of stable cell lines of Caco-2/bbe/SGLT1 cells expressing the shRNA oligomers. Knock down of protein expression was verified by Western blot analysis using specific antibodies against

NHERF1 or NHERF2. Of the three shRNAs against NHERF1 and NHERF2 tested, two of each caused at least 70% knock down of the respective NHERF while one of each had no effect.

Triple HA-tagged rabbit NHE3 was engineered into adeno-viral shuttle vector ADLOX.HTM under control of a cytomeglavirus (CMV) promoter and virus was prepared, purified and viral titers were determined as described previously [14]. Infection was typically done at day 11-12 after the cells became confluent. Caco-2/bbe-SGLT-1 cells were treated with 5 mM EGTA in serum-free Caco-2 medium for 2 hrs at 37˚C before virus exposure (10^9-10^{10} particles/ml) to both the apical and basolateral surfaces, and cells were used 48-60 hr after infection, as described [14].

Sucrose Density Gradient Centrifugation:

Jejunal segments were incubated in isotonic Na solution (138 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, mM Mg SO_4, 1 mM NaH_2PO_4, 25 mM D-mannose or α-MD-G and 20 mM HEPES, pH 7.4 with 95% O25% CO_2 gassing for 5 min at 37˚). Then total membrane protein was prepared as described previously by solubilizing in lysis buffer (20 mM HEPES, 150 mM NaCl, 1 mM Na_3VO_4, 50 mM NaF with 1%Triton-X and protease inhibitors (Sigma, St. Louis, MO))[9]. 1 mg total protein samples (1 ml volume) were loaded at the top of an 11-step discontinuous sucrose gradient of 10-30% and processed as previously described [9]. Samples from each gradient fraction were loaded onto 10% SDS-PAGE gels and monoclonal anti-NHE3 antibody (Chemicon, 1:50 dilution) was used to detect the NHE2 distribution. This commercial antibody was shown to be specific using NHE3 knockout mouse kidney (data not shown), kindly provided by G. Shull, Un Cincinnati.

Coimmunoprecipitation (IP):

IP was performed using total membrane protein from mouse jejunum or total lysates from Caco-2/bbe cells, with either anti-NHE3 antibody, prepared or described [28] or anti-HA antibody (when HA epitope tagged NHE3 was studied) by using rabbit IgG or VSV-G as a negative control. Tissue and cells were prepared as described in the sucrose gradients and surface biotinylation methods. Polyejunal NHE3 antibody was firstly crosslinked to IgG beads with Carbolinkt™ Immobilization Kit. Aliquots (2 mg of protein in 1 ml) of lysate were incubated overnight with 50 ul of either monoclonal anti-HA affinity agarose (Roche Diagnotics, Indiapolis, IN), anti-NHE3 conjugated IgG beads (Ratio between antibody and total lysate protein is 1:100) or monoclonal anti-VSV-G antibodies conjugated to agarose beads at 4˚C in a rotator (antibody conjugated agarose beads were prewashed with lysis buffer [as described above] before use). The antibody conjugated protein G-Sepharose beads were gently spun down and washed five times with lysis buffer. Bound proteins were eluted with 200ul sample buffer, and proteins were separated by SDS-PAGE and transferred to nitrocellulose. The blots were probed with polyclonal anti-NHERF2 (or anti-Flag (Abcam)), anti-Ezrin (cell signaling) or monoclonal anti-HA primary antibody (Covance Research Products), followed by fluorescently labeled secondary antibody (IRDye 800 or 680) for visualization according to the manufacturer's protocol.

Protein bands were visualized by the Odyssey system (LI-COR, Lincoln, NE).

NHERF2 and NHERF2Δ30 transfection in Caco-2-bbe cells:

cDNAs encoding human NHERF2 (residues 1-337) and its ERM-binding domain deletion mutant, NHERF2Δ30 (residues 1-307) were cloned into pcDNA3.1 Hygro+ vectors. Infection was typically done at day 11-12 after the cells became confluent. Caco-2/bbe-SGLT-1 cells were treated with 6 mM EGTA in serum-free Caco-2 medium for 2 hrs at 37˚C. 10μg DNA and 16ul lipofectamine2000 were incubated in 200μl Opti-Mem1 medium for 10 mins separately and then for another 20 mins at RT when combined.

28. Fernandez-Llama P, Andrews P, Ecelbarger CA, Nielsen S, Knepper MA . Concentrating defect in experimental nephritic syndrome: Altered expression of aquaporins and thick ascending limb NA+ transporters, *Kidney International* 1998; 54:170-179.

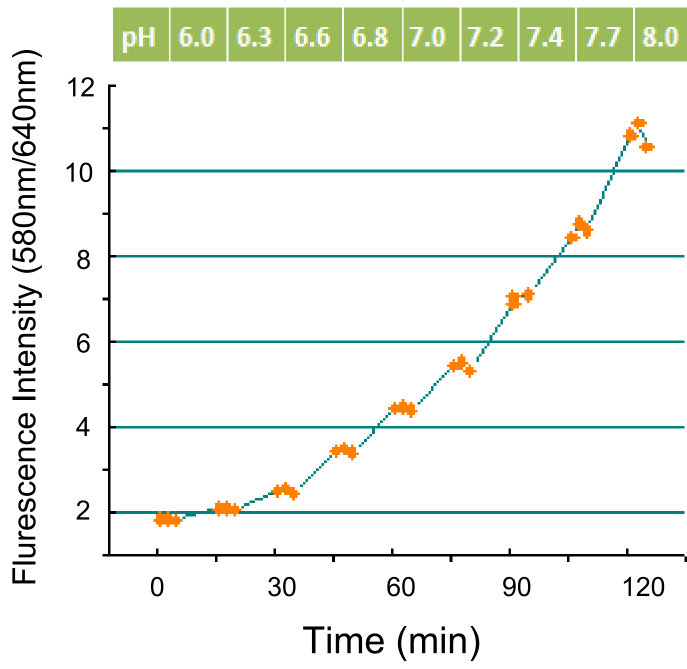
Supplementary Figure Legends

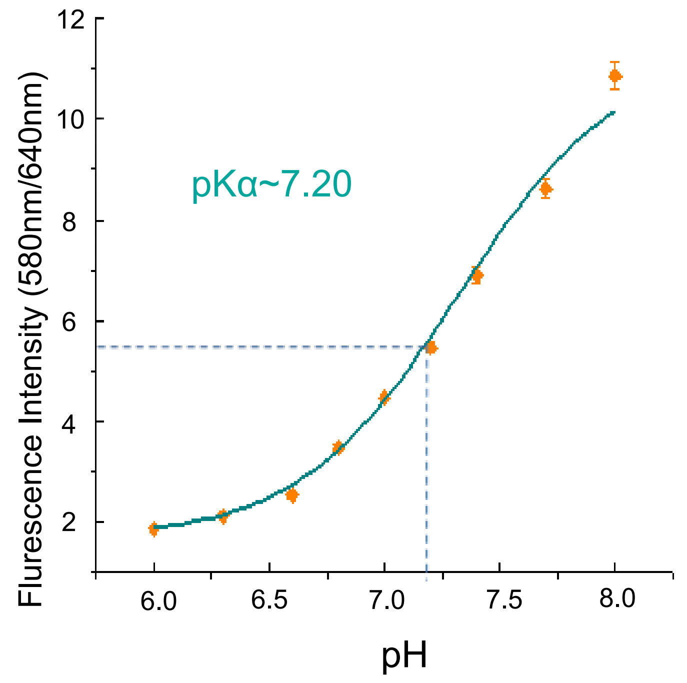
Supplementary Fig 1. Calibration of the fluorescence response of SNARF-4F AM in murine jejunum. Excised murine jejunum was mounted on coverslips, incubated with Na medium containing 20uM SNARF-4F AM, for 35 min. The emission intensity of SNARF-4F was then measured at different intracellular pHs. The jejunum was placed in a perfusion chamber on the heated stage (25 °C) of the microscope and perfused with pH controlled buffers(K+ clamp solution containing 10 μM nigericin) for 15 min at each pH shown (Panel A). For each pH, images were taken and analyzed compared to external standards, as described in Experimental Procedures. Panel B shows the correlation of the ratio of average emission fluorescence intensity correlated with pH of the buffer. Non-linear curve fitting analysis (Origin 6.0, Boltzmann curve fitting constants shown in insert) was performed to determine the apparent pKa values as shown.

Supplementary Fig 2. α-MD-G stimulation of NHE3 is associated with shifting of NHE3 into smaller protein complexes. Tissue was excised from murine jejunum and prepared for sucrose density gradient centrifugation to determine NHE3 complex size . An aliquot of each gradient fraction was separated on SDS-PAGE. NHE3 and β-actin were identified by immunoblotting on the same specimens (these are shown as upper and lower panels). α-MD-G induced a shift of NHE3 into smaller complexes. There was no change in β-actin distribution. Similar results were seen in 3 identical experiments.

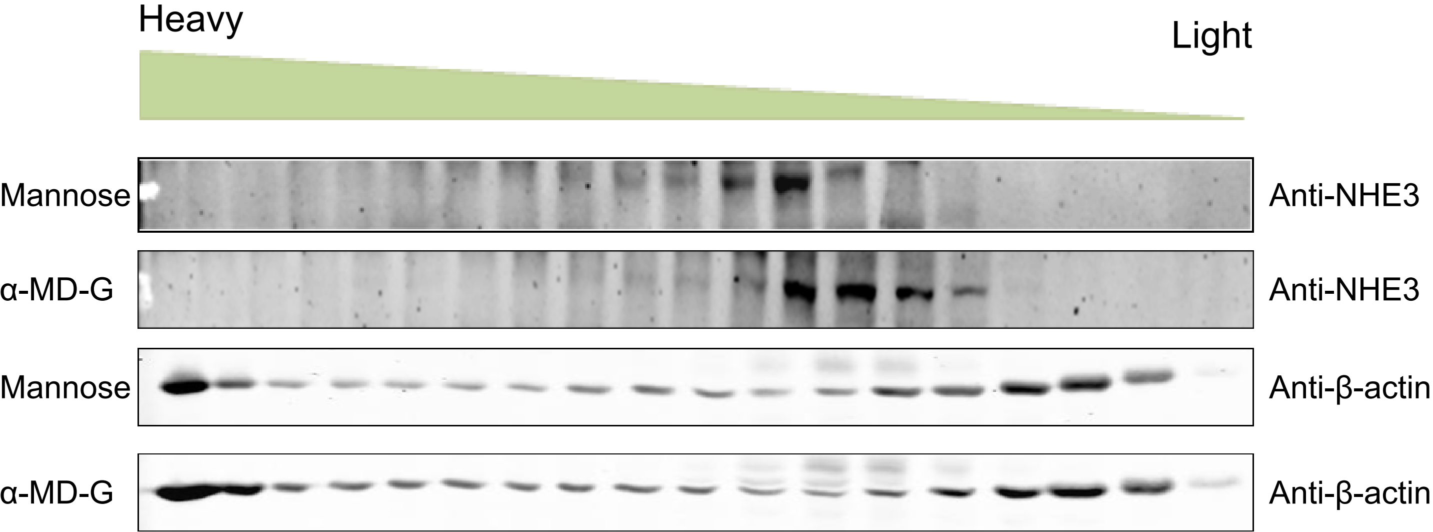
Supplementary Fig 3. Mobile fraction of BB NHE3 in Caco-2/SGLT1 cells is increased 5 min after α-MD-G exposure by a NHERF2 dependent process. FRAP was determined in polarized Caco-2 cells 14 days post-confluency and 48h after adenoviral/HA-NHE3-GFP infection. Conditions used to demonstrate that the NHE3 FRAP construct behaved like wild type, methods of calculation, and conditions to limit the contribution of trafficking to the BB NHE3 were as described [14,17]. A) Results shown are of mobile fraction from a single experiments with at least four regions of interest in at least 3 cells examined, with data for each coverslip calculated with normalization to initial fluoresence. B) Means ± SEM of three identical experiments.

Suppl. Fig 1





Suppl. Fig 2



Suppl. Fig3